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**Original Article**

# Herb Preparations Improve the Viability of Hippocampal Cells Suppressed by Amyloid Beta (1-42) Peptide

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## ABSTRACT

The aggregated amyloid peptides (Aβs) are considered as the hallmarks of Alzheimer's disease. We studied the ability of plant preparations, decreasing the aggregation state of amyloid peptides, to improve the viability of different type hippocampal cells, preliminarily suppressed by aggregated Aβ(1-42) peptide. The hippocampal cells were stained by antibodies against rabbit anti-rat Neurofilament (NF), glial fibrillary acidic protein (GFAP) and Nestin, which are specific for neuronal, glial and multipotent neural stem cells (NSCs), respectively. The aggregated Aβ(1-42) decreased *in vitro* the viability of all cell types down to <20 %. In the presence of phenol glycosides fraction from rose petals, ethanol extracts from sorrel leaves and melilot, the viability of GFAP-positive cells was restored up to ~170-150% of Aβ(1-42)-controls. The viability of Nestin-positive cells was restored by phenol glycosides fraction from rose petals and ethanol extract from sorrel leaves up to 210 and 230%, respectively, ($p<0.05$). Because the glial (GFAP-positive) and neural stem cells (Nestin-positive) can be reprogrammed into neurons, we can suggest sourcing therapeutics for neurodegeneration prevention and/or treatment from these plants.

**Keywords:** amyloid peptide cytotoxicity; hippocampal cell regeneration; immunocytochemistry, plant preparations

## INTRODUCTION

In some cases, protein aggregation occurring in living organisms is a natural phenomenon. However, the non-physiological aggregation and misfolding into insoluble amyloid fibrillar structure of many proteins (among them: beta-amyloid, alpha-synuclein, huntingtin and ataxin, superoxide-dismutase 1, tau and amylin) is responsible for several neurodegenerative pathologies (Alzheimer's, Parkinson's and Huntington's diseases, Amyotrophic Lateral Sclerosis, Frontotemporal Lobar Degeneration, Type 2 Diabetes, etc.).

Alzheimer's disease (AD) is a neurodegenerative disorder in aging population, characterized by progressive impairment of memory, cognition and behavioral functions. It is associated with neuronal cell loss and synaptic dysfunction [1,2], which are mainly conditioned by deposition of tangles formed of amyloid-β peptides (Aβs) and tau proteins [3]. Oligomeric and fibrillar aggregates generated by these peptides are among the principal components of amyloid plaques found post mortem in AD brain [4]. Therapeutic agents, abolishing the formation of fibril aggregates, have not been found [5,6].

One of the current approaches in the treatment of AD is the inhibition of the Aβs aggregation and the promotion of the decay and destruction of the formed aggregates by natural agents [7,8,9]. The results of *in vitro* researches indicated that resveratrol, an active polyphenol in many plants, may directly bind to Aβ42 interfering its aggregation, changing oligomer conformation and attenuating cytotoxicity [10,11]. *Rosa damascena* is rich in effective flavonoids and possesses multifunctional and multitargeted characteristics. Its extract reversed the behavioral deficit in rat model of Aβ-induced AD, manifesting a potential for prevention and treatment of cognitive dysfunction [12]. Kim and Oh examined the current researches on developing herbal anti-AD agents, focusing on their advantages, discussing the pros and cons. They concluded that herbal medicine can be useful in AD-prevention, and "synergistic therapeutics, combining

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conventional medicine and herbal medicine for AD, may emerge in the not-so-distant future" [6].

Earlier, we have shown the ability of the extracts from several Armenian highland plants and their fractions to inhibit the aggregation of islet peptide hormone, amylin, to disaggregate its preformed aggregates, and to protect the cultured mouse pancreatic β-cells against cytotoxicity of aggregated amylin [13,14]. The *in vitro* inhibition by plant preparations (PPs) of aggregation and disaggregation of the preformed aggregates of Aβ42 and Aβ40 was demonstrated by using Thioflavin-T fluorescence technique [15]. For some PPs, the $IC_{50}$ values in these processes were evaluated.

On the other hand, a long-held "truth" that the adult mammalian brain is mainly a post-mitotic structure lacking the ability to regenerate neurons was overturned in the past century. In the adult brains, discrete "neurogenic" regions have been identified [16,17]. New neurons in these regions originate from a residential population of adult neural stem cells [18,19,20], which arise under pathological conditions or after injuries [21,22]. The possibility of replacing the neurons lost after nerve injuries or diseases by direct reprogramming of reactive glial cells (astrocytes and NG2 cells) into functional neurons, was suggested [23,24]. Understanding the basic mechanism of the adult neurogenesis regulation and contribution to brain functions is important both for basic biology and clinical use for repairing the unwell, injured and aged central nervous system [25].

The present work looked for the answer whether the plant preparations, which decrease the aggregation state of amyloid peptides, can contribute to restoration of the suppressed viability of neuronal cells. To this end, firstly, the hippocampal cell culture was stained with different specific primary antibodies. Then, the toxicity of aggregated Aβ42 toward all the stained cell types was registered. Afterwards, the beneficial effects of several plant preparations on at least two hippocampal cell types (glial and neural stem cells) were found.

## MATERIALS AND METHODS

**Materials:** Thioflavin-T (ThT), RPMI-1640 and supplements were purchased from Sigma Ltd, USA; Aβ40 and Aβ42 peptides were purchased in "China peptide" (China). Antibodies for immunocytochemistry were purchased from Neuromics (USA). All other chemicals were of the highest purity.

**Equipment:** Spectral measurements were performed on Specord M-40 UV-VIS spectrophotometer (Germany) and Perkin-Elmer MPF-44A spectrofluorometer (USA). The light and fluorescence microscope models: BH- 2RFCA, Olympus, with a digital camera model c35AD-4, Olympus; Boeco BM-800 (Germany) with Blue Exciting Light Filter; and Crocus 5MP MCX100 (Micros, Austira) were used.

**Plant material:** The leaves of grape *(Vitis vinifera)* and sorrel (*Rumex Confertus)*, rose petals *(Rosa damascena*) and underground part of melilot (*Melilotus officinalis)* were collected in Armenian highland and dried in the shade. A voucher specimen has been deposited in the herbarium of the Botanical Department of Yerevan State University (Dr. Narine Zaqaryan). The preparation of the plant extracts, the separation of fractions and examination of the constituents by usual chemical analysis methods were described earlier [26]. After drying by evaporation, the plant preparations stored at -18$^{o}$C. In the experiments, the aliquots of stock solutions in 70% ethanol/water were added to the cell incubation medium up to the desired concentration. The final concentration of ethanol in the assay mixture was below of nontoxic, 1 % level.

**Peptide preparation:** The preparation of the solutions and aggregates of Aβ40 and Aβ42 peptides were described earlier [15,27,28].

**Hippocampal cell culture:** The hippocampal cell culture from hippocampus of adult rats was obtained as described earlier [28]. The experiments involving the laboratory animals were approved by Ethics Committee of Yerevan State Medical University after M. Heratsi, No 7-26.04.2012: Research is not contrary to the Directive 2001/20/EC of The Legal Aspects of Research Ethics and Science in European Community.

**Immunocytochemistry:** Hippocampal cells were cultured in Poly-D-Lysine coated Nunc EasY Flasks. On the 4$^{th}$ day, the adhered cells were removed by trypsinisation (0.05% trypsin, 0.5 mM EDTA, pH 8.0). Cells were fixed on microscope slide by 3.7 % paraformaldehyde in PBS followed by methanol treatment. To block a nonspecific antibody binding, samples were pre-treated with goat serum (Sigma) for 30 minutes. To determine the types of cells, constituting the obtained cell culture, the different aliquots of the culture were incubated for 60 min at room temperature with the primary antibodies against rabbit anti-rat Neurofilament (NF), chicken anti-rat GFAP and Nestin. These antibodies are specific for neuronal, glial and multipotent neural stem cells,

respectively. Afterwards, the cells were treated with FITC-conjugated secondary antibodies (goat anti-rabbit or anti-chicken) [29].

A fluorescent assay with ethidium bromide (EB) staining [30] was used to display the total cell count. Shortly: a drop of cell suspension on the microscope slide was dried in air, fixed with ethanol for 10 min, washed with distilled water and dried. Then it was covered with EB (50 μg/ml) for 5 min; the excess of the dye was washed out. Slides were examined and photographed using fluorescence microscope with Blue Exciting Light Filter system at magnification ×75. The adhered cells were inspected in the inverted culture flasks at magnification ×500 using light microscope. The images were captured using Canon PC1200 digital camera.

**Statistical analyses:** The statistical analyses of data, obtained at least in three independent experiments, were performed using the InStat software, version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was accepted for one-tailed p-value <0.05. The results are expressed as means ± SEM (standard errors of means).

## RESULTS AND DISCUSSION

**The cytotoxicity of aggregated amyloid peptides toward hippocampal cells:** Our experiments confirmed the cytotoxicity of aggregated Aβs toward hippocampal cells, described by many researchers. The cultivation of hippocampal cells in the presence of different concentrations of aggregated and non-aggregated Aβ40 and Aβ42 demonstrated higher toxicity of the aggregated versus the non-aggregated peptides. The toxicity of Aβ42 was more pronounced than that of Aβ40: we observed nearly identical inhibition of the viability of cells at about 10-fold lower concentration of Aβ42.

In several independent experiments, the *in vitro* cytotoxicity of aggregated peptides was confirmed by cultivation of hippocampal cells in their presence. The number of living cells in the control samples ($59.8\times10^5$ ± 4.0, n=7) after a 3-day cultivation in the presence of 2 μM aggregated Aβ40 decreased on average to 22.7% ($13.6\times10^5$ ± 1.9, n=5). In the presence of 0.2 μM aggregated Aβ42, the number of cells decreased on average to 20.2% ($12.1\times10^5$ ± 1.5, n = 6) ($p<0.0001$). Hence, our experiments have shown that the aggregated Aβs in the used concentrations diminished the viability of hippocampal cells down to nearly 20% of control.

It is known, that Aβ40 and Aβ42 contribute to the AD process differently. Aβ40 is a more common form of the two, while Aβ42 is more fibrillogenic and pathogenic [31]. Based on this knowledge and on our findings, we studied the influence of aggregated peptides on the hippocampal cells, using the concentrations of 2 μM for Aβ40 and 0.2-0.4 μM for Aβ42.

Fig.1 compares light microscopy images of the cell suspensions after a 3-day cultivation in the absence (A) and presence of 0.4 μM aggregated Aβ42 (B). Distortion of cell membranes in the medium, containing aggregated Aβ42, is obvious.

**Protection of viability of hippocampal cells by plant preparations:** The ethanol extracts from melilot (M), rose petals (RP), sorrel leaves (SL), grape leaves (GL) and the fractions of phenol glycosides (PhG) isolated from GL and RP, flavonoids (Fl) from SL, coumarins (Cm) from GL and SL in the concentration range 3-300 μg/ml *per se* had no viability-lowering effects on hippocampal cells. Moreover, most of these plant preparations protected the viability of cells in the presence of aggregated Aβs ($p<0.05$).

The calculated $IC_{50}$ values in the protection of hippocampal cells from cytotoxic action of Aβs for some of plant preparations were rather low. In the case of Aβ42, these values were in the range of 14-26 μg/ml for the extracts and 2.1-8 μg/ml for the fractions; in the case of Aβ40 they were between 0.5-27 μg/ml and 0.2-20 μg/ml, respectively.

**Differently stained hippocampal cells in the presence of aggregated Aβ42:** Different aliquots of cell culture were stained (see Materials and Methods) by ethidium bromide and primary antibodies against NF, Nestin and GFAP. Fig. 2 shows the types of cells, constituting the cell culture, isolated from hippocampus of adult rats and used in our experiments.

The staining technique was applied to clarify which of the cell types responds to the cytotoxic action of the aggregated amyloid Aβ42 peptide. Table 1 shows the counts in the differently stained aliquots of cell culture. The first row presents the cell counts for all the cell types "as isolated". The second row shows a moderate decrease of all cell types after a four-day cultivation in a usual culture medium. A concurrent four-day cultivation in the presence of 0.4 μM aggregated Aβ42 (row 3) evidenced a decrease of all the cell types down to 16-18% (row 4). The EB-stained cell counts decreased to ~19%, close to the above described decreasing of viability of hippocampal cells during

cultivation in the presence of aggregated Aβs (~20%).

**The repair of cell viability, suppressed by Aβ42, in the presence of plant preparations:** In the next experiment, the cell culture was divided to six aliquots after a two-day cultivation in the presence of 0.4 μM aggregated Aβ42. The PhG fraction from RP and ethanol extracts from GL, RP, M, SL were added into five of them up to concentration of 200 μg/ml. The cultivation continued for 2 more days. Then, the staining technique was applied to find out which of the cell types responded to the exposure to plant preparations after suppressing by aggregated Aβ42.

Fig. 3 presents the cell counts of differently stained hippocampal cells in this experiment. The percentage of cell counts of the respective Aβ42-controls is shown. The presented data indicate that several herbal preparations did not only neutralize the effect of Aβ42, but also stimulated the growth of some cell types.

A two-day cultivation in the presence of RP PhG and extracts from SL and M demonstrated a statistically significant increase (up to 176%, p=0.0229; 171%, p= 0.0150; 148%, p=0.0419, respectively) in the number of GFAP-positive glial cells relative to Aβ42-control. It is obvious that the PPs restored the viability of said cells.

A significant elevation of Nestin-positive cells was observed in the presence of SL (up to 230%, p= 0.043) and RP PhG fraction (210 %, p=0.05). They are self-renewing, multipotent neuronal stem cells (NSCs), differentiating into neurons, astrocytes and oligodendrocytes.

The content of NF-positive cells (neurons) did not differ statistically from Aβ42-control. The plant preparations did not have a direct positive effect on neurons - at least, in the conditions of our experiment.

Nevertheless, the results of immunocytochemical investigation of hippocampal cells suggested that some of the used PPs support the proliferation of, at least, glial and multipotent neuronal stem cells even in the presence of the toxic aggregated Aβ42. We can assume that a longer cultivation of Nestin-positive cells with SL and RP PhG can result in restoration of all the hippocampal cells, NF-positive neurons including [32]. It is possible that the *in situ* and/or *in vivo* restoration of functional neurons in the presence of these plant preparations also can occur via reprogramming of the reactive glial and NSCs cells [23,24].

We can conclude that the SL, M and RP PhG, supporting the GFAP- and Nestin-positive cells, are beneficial for all the hippocampal cells. They can be considered as neuron-saving remedies against the toxic action of amyloid peptides. Of course, further investigations are required to clarify the mechanisms of the PPs effects observed in this study. Mechanisms, proposed for the development of stem cell biology and regenerative medicine using natural and chemical small molecules [33,34], may be realized.

## Conclusion

Our data confirm that the aggregated forms of Aβ40 and Aβ42 peptides *in vitro* are more cytotoxic toward hippocampal cells than their non-aggregated forms, Aβ42 being ten-fold more toxic. Rose petals, melilot, grape and sorrel leaf extracts and their fractions effectively protect the hippocampal cells in the presence of aggregated Aβs. Besides, the ability of several plant preparations to restore the viability of hippocampal cells, depressed by amyloid peptides, was shown.
The studied plants grow all over the world and are widely used in eastern cuisine. To the best of our knowledge, this is the first report on the neuroprotective efficacy of melilot, grape and sorrel leaf extracts. They can be considered as agents, supporting neurogenesis, and can serve as valuable sources for therapeutics with the potential to treat neurological and other amyloid disorders.

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**Table 1. Cell counts per view point of differently stained hippocampal cell culture**

| Cells | | EB (total) | NF (neurons) | NESTIN (NSCs) | GFAP (glial cells) |
|---|---|---|---|---|---|
| upon isolation | | 67.8±2.9 | 19.5±1.5 | 16.5±1.2 | 32.5±0.6 |
| four-day incubation | (a) without Aβ42 | 53±3.6 | 12.8±2.0 | 14.3±0.9 | 29.5±2.5 |
| | (b) with Aβ42 | 10±0.9*** | 2.3±0.6*** | 2.3±1.1*** | 5.3±0.9** |
| | (b) as % of (a) | 18.9 | 17.7 | 15.8 | 17.8 |

***P<0.001; **P<0.01; veracity relative to "without Aβ42"

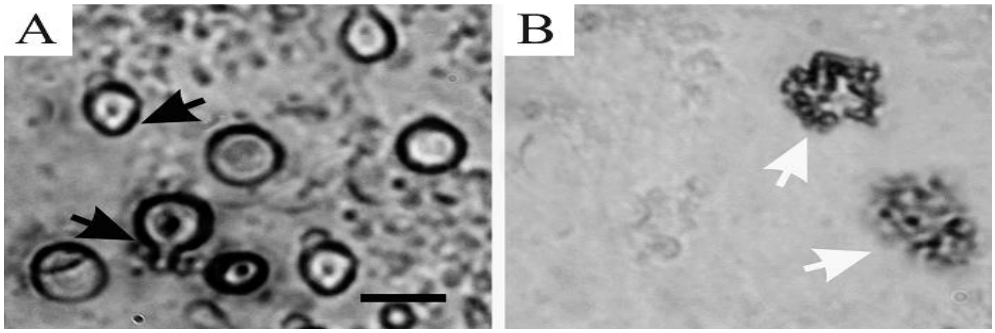


**Figure 1:** The toxicity of aggregated Aβ42 towards hippocampal cells: the cell suspensions after a 3-day cultivation in the absence (A) and presence (B) of 0.4 μM aggregated Aβ42. The black and white arrows indicate, respectively, the integral and degraded cells; scale bar 10 μm applies to both frames.

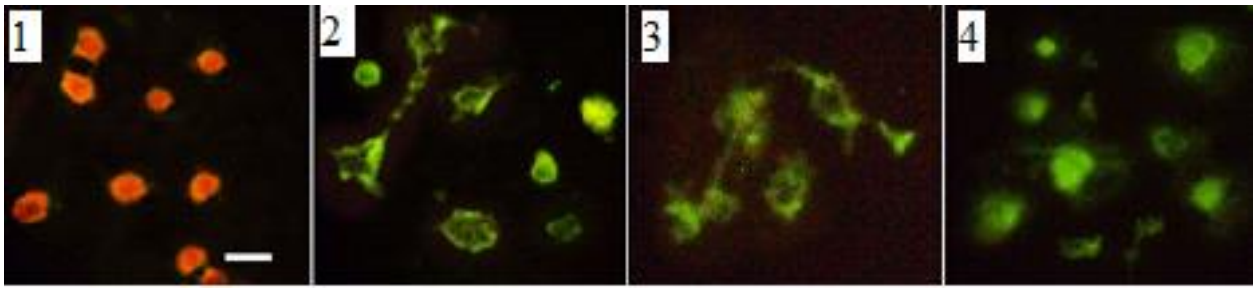


**Figure 2:** The images of hippocampal cells stained by ethidium bromide (1, total) and primary antibodies against NF (2), Nestin (3) and GFAP (4), specific for neuronal, multipotent NSC and glial cells, respectively. Scale bar 20 μm applies to all frames.

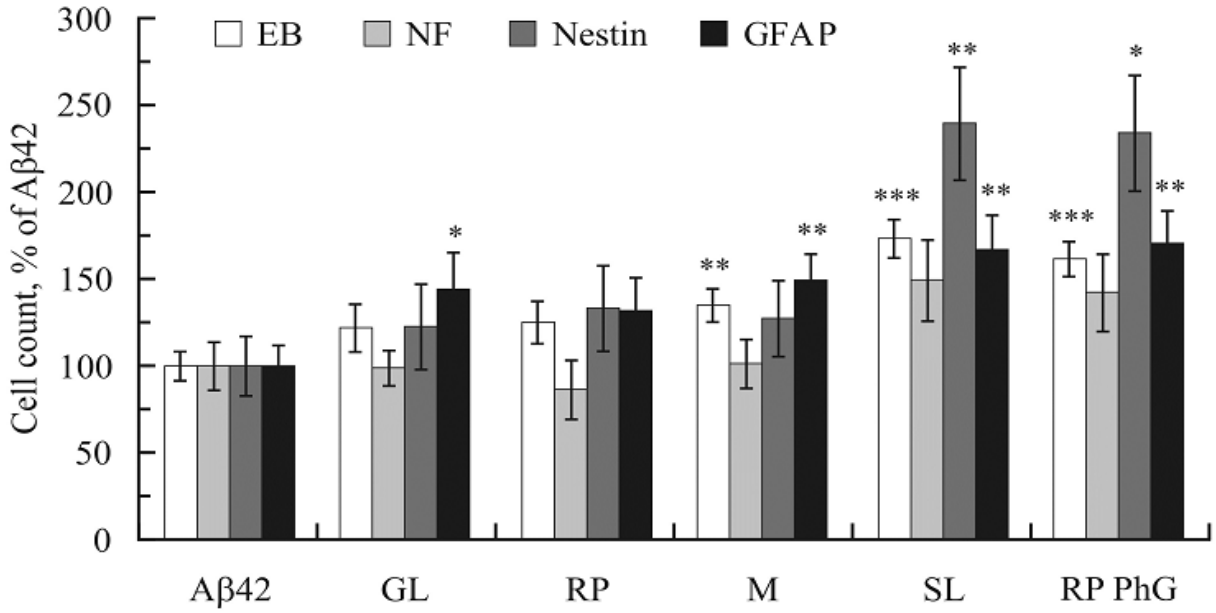


**Figure 3:** The viability of different types of hippocampal cells (neuronal, NF; NSC, Nestin; glial, GFAP) after incubation during four days in the presence of 0.4 μM aggregated Aβ42. The last two days cultivation medium contained PhG fraction from RP and ethanol extracts from GL, RP, M and SL in concentration of 200 μg/ml. The viability is expressed as percentage of Aβ42-controls. The bars indicate ± SEM as % of appropriate values. ***P<0.001, **P<0.01, *P<0.05 - the veracity relative to Aβ42-controls.

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